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The Consumption of Antithrombin III During Coagulation, Its Consequences for the Calculation of Prothrombinase Activity and the Standardisation of Heparin Activity

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Summary

The decay rate of thrombin in plasma is shown to be linearly proportional to the concentration of antithrombin III (AT III), not only in the absence but also in the presence of heparin. This is a consequence of partitioning of heparin between AT III and other plasma proteins.

In previous articles we calculated the prothrombin converting activity assuming a fixed concentration of AT III. Since AT III is consumed during the clotting process, prothrombinase activity is more accurately approximated using an algorithm that counts with the decrease of the AT III concentration. It is shown this leads to higher prothrombinase activities. The (absence of) inhibition of prothrombin conversion by prothrombinase in the presence of heparins found with the previous method is also found using the new algorithm.

From the results presented it is evident that characteristic parameters of heparin action have to be normalised to the AT III concentration. On this basis we define a Standard Independent Unit of the antithrombin activity of heparin.

Introduction

The understanding of the action of heparins in plasma requires a detailed analysis of the influence of heparin on thrombin decay. We have previously described how we determined the pseudo first-order decay constant (k_{dec}) of endogeneously generated thrombin in plasma, in the absence and in the presence of heparins. In the course of the thrombin generation process we stopped prothrombin conversion via inhibition of factor Xa with soybean trypsin inhibitor and determined the velocity of decay of the remaining thrombin (1, 2). We showed that the global decay constant thus obtained is the sum of the decay constant (k_2) caused by α_2 -macroglobulin (α_2 M) and an α_2 M-independent decay constant (k_1). The latter consists of an AT III-independent part (~10%) and an AT III-dependent part (~90%). The AT III-independent part is caused by various inhibitors, mainly by α_1 -antitrypsin (1), but not by heparin cofactor II (HC II) because, at heparin concentrations <2 U/ml, as used in this article, the stimulation of HC II is negligible (3). We therefore can describe the increase of thrombin decay brought about by the addition of heparin in terms of the acceleration of AT III dependent inactivation.

The level of AT III antigen in serum is significantly smaller than the initial plasma level (4, 5) because AT III is consumed during the coagulation process. In this article we wanted to investigate in detail whether the decrease of AT III during the

coagulation process does affect the decay constant of thrombin in the presence of heparin.

The answer is not obvious a priori. In previous work we observed no variation of k_{dec} between 4 and 7 min after the start of thrombin generation, i. e. after the thrombin peak concentration has occurred. In the absence of heparin one might expect k_1 to decrease proportionally with the AT III concentration during the coagulation process since k_1 is the product of a second order decay constant k_1' and the AT III concentration. In the presence of heparin the question is more complicated. The 2 μ M of AT III present in plasma represent a large molar excess over 1 U/ml of heparin (1 U/ml \approx 110 nM AT III binding standard heparin). The heparin-catalysed interaction of AT III and thrombin was shown to be saturable with respect to both AT III and thrombin and the apparent dissociation constant of the functional AT III-heparin complex is ~160 nM (6–10). This means that the normal plasma level of AT III could be expected to bind virtually all heparin up to 1 U/ml and even higher. The rate of thrombin inactivation then should be proportional to the concentration of heparin only, and not depend any more on the AT III concentration.

In plasma, however, other heparin binding proteins may compete for heparin. The outcome of this competition is dependent upon the affinity constants and concentrations of all heparin binding proteins and no reasonable guess can be made on the influence of AT III concentration on k_{dec} . Therefore we reinvestigated in detail the course of k_{dec} during the coagulation process and its dependence on AT III and heparin concentration.

Materials and Methods

Chemicals

The chromogenic substrate for thrombin was H.D-Phe-Pip-Arg-pNA (S2238) from Kabi, Sweden. The 4th International heparin standard (4th I. S. H.) was from the National Institute for Biological Standards and Control. Reptilase was obtained from Laboratoires Stago (Asnières, France), a solution was made according to the instructions of the manufacturer. Soybean trypsin inhibitor (batch No. 43 F-800) was obtained from Sigma (St. Louis, MO).

Blood and Products

Blood from 10 healthy donors was collected on 0.13 M trisodium citrate; nine parts of blood to one part of citrate solution.

Platelet poor plasma (PPP) was obtained after two centrifugations at $900 \times g$, 15° C for 15 min and a third centrifugation at $23,000 \times g$, 4° C, for 1 h. PPP was pooled and stored at –80° C. It was checked that the clotting factors and the antiproteases were in the normal range.

Defibrinated plasma was obtained by mixing an aliquot of plasma with 1:50 volume of a reptilase solution, letting a clot form for 5 min at 37° C and keeping the clotted plasma at 0° C for 10 min. The fibrin clot formed was discarded by winding it on a small plastic spatula. The concentration

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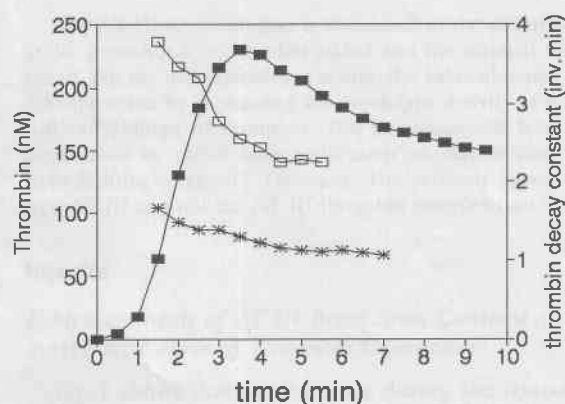


Fig. 1 The course of the endogenous thrombin decay constant during the thrombin generation test. The total constant ($k_1 + k_2 + k_3$) is given, as obtained after inhibiting thrombin generation by addition of SBTI (16.5 μM final concentration). ■ Thrombin generation test in normal plasma. * k_{dec} in normal plasma. □ Idem with 0.03 U/ml heparin

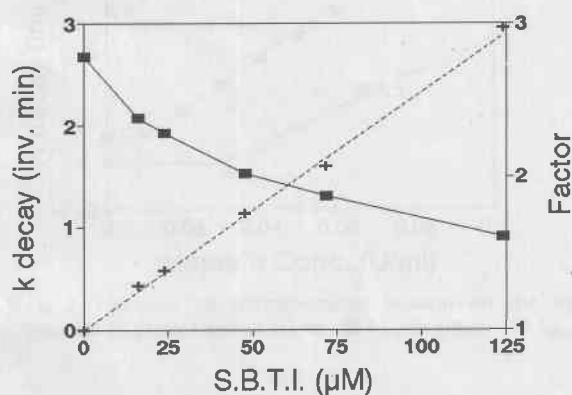


Fig. 2 Influence of SBTI on the thrombin decay constant in plasma. ■ k_{dec} (left ordinate); + ratio k_{dec} with SBTI/ k_{dec} without SBTI (right ordinate)

Table 1 The influence of SBTI on thrombin decay

	No SBTI	SBTI	Ratio
Plasma	2.77 (15.0)	2.08 (20.1)	1.33
Plasma + 0.04 U/ml hep.	4.67 (9.1)	3.47 (11.9)	1.33
Plasma + 1.25 $\mu\text{g}/\text{ml}$ DS	4.16 (10)	3.12 (13.3)	1.33
Serum	1.63 (25.5)	1.19 (35.3)	1.37
Serum + 0.1 U/ml hep.	3.76 (11.1)	2.83 (14.7)	1.32

The total decay constant ($k_1 + k_2$) is given in min^{-1} , in brackets the half life times in s. DS = dermatan sulfate.

of factors II, VII, VIII, IX, X, XI and XII did not significantly change by the reptilase treatment (1).

Serum is the material left after plasma has been activated as in a thrombin generation test (see below).

Euglobulin Fraction

The euglobulin fraction is obtained from defibrinated pooled plasma by acid precipitation (pH 5.2) at low ionic strength as described in Josso and Prou-Wartelle (11). The precipitate is collected by centrifugation and dissolved in half the initial plasma volume of 21.6 mM trisodium citrate in buffer A. In order to obtain thrombin, thrombin generation is triggered by

adding 0.25 vol of diluted human brain thromboplastin in 100 mM of CaCl_2 to 1 vol of euglobulin fraction.

Human brain thromboplastin was prepared according to Owren and Aas (12).

Determination of Thrombin Generation in Plasma

To 240 μl of defibrinated plasma are added 60 μl of 50 mM Tris-HCl, 100 mM NaCl, 0.5 mg/ml ovalbumin, pH 7.35 (buffer A), containing heparin at the desired concentration. Thrombin formation is started by the addition of 60 μl of a solution containing 100 mM of CaCl_2 and human brain thromboplastin, diluted so as to clot the uninhibited, non-defibrinated reaction mixture in 70 s (final dilution $\sim 1:240$). At intervals a 10 μl aliquot of the mixture is sampled into a disposable plastic cuvette containing 465 μl of 50 mM Tris-HCl, 100 mM NaCl, 20 mM EDTA, 0.5 mg ovalbumin/ml, pH 7.90 (buffer B) and 25 μl of S2238 (4 mM) and prewarmed for at least 5 min at 37° C. After about 120 s the reaction in the cuvette is stopped by adding 300 μl of concentrated acetic acid. The precise moments of sampling and stopping were recorded on a personal computer by means of pushbutton equipped pipettes. Optical densities are read at 405 nm in a dual wavelength (405 nm–536 nm) dedicated instrument constructed in our workshop. From the optical density and the start and stop times, the amidolytic activities (mOD/min) were calculated. The amidolytic activity is the result of a) free thrombin and b) α_2 macroglobulin bound thrombin ($\alpha_2\text{M-T}$). At the end of the coagulation process a stable endlevel of amidolytic activity is obtained, due to the $\alpha_2\text{M-T}$ complex formed. k_2 in our batch of normal plasma, determined according to Hemker et al. (1), equalled 0.23 min^{-1} and was independent of the heparin concentration.

The Pseudo First Order Rate Constant of Thrombin Inactivation

Endogenous thrombin. Defibrinated plasma (120 μl) was incubated with 14 μl of buffer A. Thrombin generation was started as described in the previous section. At a timepoint when the concentration of thrombin was sufficiently high, 6 μl of Soybean Trypsin Inhibitor (SBTI) solution (10 mg/ml) and 10 μl of buffer containing the required amount of heparin were added. SBTI at this concentration was shown to block the activity of prothrombinase completely and instantaneously and not to influence the activity of thrombin on chromogenic substrate (1). At suitable time intervals (2–10 s) after addition of the SBTI solution, 10 μl aliquots were drawn for measurement of thrombin concentrations, as described above.

Exogenous thrombin. A mixture of 120 μl of defibrinated human plasma and 40 μl of buffer A with or without heparin was prewarmed for 2 min at 37° C, then 20 μl of a thrombin preparation were added to so as to obtain a final concentration of 100 nM. The disappearance of thrombin was then measured as described above.

In order to approach the experimental conditions of endogenous thrombin decay as closely as possible, we have not used purified thrombin but thrombin that was freshly generated either in a euglobulin fraction (see above) or in plasma (same conditions as in the thrombin generation experiments).

In both methods the amidolytic activities (C_t) as obtained in the experiment, were fitted to the three parameter curve $C_t = C_\infty + (C_0 - C_\infty) e^{-k t}$, where $k = k_{\text{dec}} = k_1 + k_2$ (see introduction). C_0 is the initial amidolytic activity. C_∞ is the residual, steady end-level activity due to the $\alpha_2\text{M-T}$ complex. In our hands, the standard error of a single estimation is below 7% as long as half life times exceed 4 s ($k_{\text{dec}} = 10 \text{ min}^{-1}$). At shorter half life times it increases proportionally with k_{dec} , with about 1% per min^{-1} .

Determination of AT III in Plasma and in Serum

The functional assay is essentially the titration of an unknown amount of AT III with a known, excess amount of thrombin. 100 μl of a suitable dilution of the sample (1:100 to 1:800) in buffer B containing 2 U/ml standard heparin, with 100 μl of a 20 nM thrombin solution were incubated at 37° C in a plastic spectrophotometer cuvette during 10 min. Then 300 μl of 330 μM S2238 in buffer B were added, and the residual thrombin induced amidolytic activity was measured at 405 nm in a spectrophotometer thermostated at 37° C. This amidolytic activity was converted into nM thrombin via a reference curve obtained with active site titrated thrombin.

The AT III concentration is calculated as the difference between the initial thrombin concentration added and the residual thrombin activity found. For the measurement in serum, the value obtained is corrected for α_2 M-thrombin by subtracting the amidolytic activity of serum, measured without addition of thrombin. The immunological level of AT III was determined by radial immunodiffusion on commercial diffusion plates from Behringwerke AG, Germany. The antibody appeared to recognise free AT III and not the AT III-thrombin complex (see Discussion).

Results

I. Measurements of AT III Breakdown Constant of Thrombin during the Course of Thrombin Generation

Fig. 1 shows that k_1 decreases during the course of thrombin generation. The presence of heparin (0.03 U/ml) added together

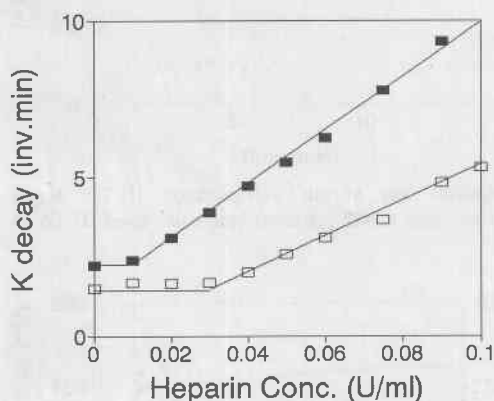


Fig. 3 Influence of unfractionated heparin on the thrombin decay constant in plasma and in serum. ■ k_{dec} in plasma; □ k_{dec} in serum

Table 2 Comparison of AT III determinations in plasma and in serum

Method	Plasma	Serum	Ratio
Antigenic AT III (%)	112 ± 13	69 ± 8	1.62
Functional AT III (μM)	2.24 ± 0.11	1.49 ± 0.07	1.50
$k_1 = k_1' [\text{AT III}]$ (min ⁻¹)	3.379 ± 0.246	2.274 ± 0.090	1.49
$k_1/[\text{AT III}] = k_1' \text{ (min}^{-1}/\mu\text{M)}$	1.508	1.522	0.99

The antigenic AT III values are obtained in 33 individual plasmas from normal volunteers and expressed as % of the "normal plasma" that accompanied the plates. The functional AT III and k_1' values were obtained in normal pool plasma (mean of 12 experiments) and corrected for the 2:3 dilution in the experimental system.

Table 3 Comparison of old and new calculations

[Hep] (U/ml)	[IIa] (nM)	Inhibition %	Prothrombinase (nM/min)	Inhibition %
A				
0.00	147.90	0.00	341.58	0.00
0.02	119.50	19.20	309.57	9.40
0.03	90.30	38.90	319.18	6.60
0.04	79.00	46.60	333.77	2.30
0.05	58.10	60.70	350.47	-2.50
B				
0.00	143.00	0.00	234.20	0.00
0.02	115.30	19.40	209.01	10.70
0.03	87.40	38.90	215.54	8.00
0.04	76.40	46.60	235.09	-0.40
0.05	56.07	60.80	217.32	7.20

Prothrombinase activities calculated A) counting with AT III consumption, and B) using pseudo first order AT III dependent decay constants.

with the SBTI increases the decay constants found but the decrease in the course of the experiment remains. Only a small change is observed after the peak of the curve, in contrast to the first minutes of the test, when the constant decreases rapidly. This explains why we considered the decay constant not to vary on basis of previous experiments carried out after the peak only (1). The experiments carried out on endogenous thrombin require a sufficient amount of thrombin to be generated. They cannot be performed in plasma or during the first minute of a thrombin generation test, nor in serum or during the last minutes. In order to obtain data on plasma and serum one has to add exogenous thrombin. Because we wanted to remain as close as possible to endogenously generated thrombin we prepared thrombin by recalcification of the euglobulin fraction of plasma, a crude fraction containing all the clotting factors but none of the inhibitors (11).

II. Comparison of the AT III Constant of Exogenous or (Endogenous) Native Thrombin

Before using the euglobulin thrombin preparation it was necessary to compare the decay constant of this exogenous thrombin to that of native thrombin generated during the test. To this end we compared the decay constant of euglobulin-thrombin to that of the thrombin generated in defibrinated plasma and added to non-triggered defibrinated plasma. No significant difference was found between the two values: the half life time of euglobulin-thrombin under these conditions is 15.4 ± 0.4 s, that of thrombin added from triggered plasma is 15.1 ± 0.4 s. The corresponding overall decay constants ($k_{dec} = k_1 + k_2$) are resp. $2.699 \pm 0.065 \text{ min}^{-1}$ and $2.784 \pm 0.073 \text{ min}^{-1}$ ($n = 8$). These values are not corrected for the 2:3 dilution of plasma in our experimental system.

III. Soybean Trypsin Inhibitor and Thrombin Breakdown

Using exogenous thrombin we have measured k_1 in serum. It turned out to be significantly higher than was to be expected from previous values obtained near the end of the thrombin generation curve (1, 2). It appears (Table 1), that the presence of SBTI (16 μM) decreases k_{dec} by a factor 1.33, both in plasma and in serum, in the presence and in the absence of heparin and of dermatan sulfate.

As can be seen in Fig. 2 the effect of SBTI on the thrombin breakdown constant increases with the SBTI concentration. The ratio of k_{dec} in the presence and in the absence of SBTI varies linearly with the SBTI concentration. This is compatible with a reversible binding between SBTI and thrombin with an apparent K_d of $\sim 70 \mu\text{M}$. The difference between k_{dec} of endogenous and of exogenous thrombin therefore appears to be explained by the addition of SBTI necessary for the inhibition of prothrombin conversion if the decay of endogenous thrombin is to be measured.

IV. The Thrombin Breakdown Constant in Plasma and in Serum in the Absence and in the Presence of Heparin

The AT III-dependent decay constant of thrombin in normal undiluted plasma is 1.5 times higher than that in the corresponding serum (Table 2). Both in plasma and in serum we observed (Fig. 3) that low concentrations of heparin do not influence the decay constant. The threshold is at 0.01 U/ml in plasma and at 0.04 U/ml in serum (see also discussion). Above the threshold value k_{dec} depends linearly on the heparin concentration. The slope of these lines describes the dependency of k_1 on the heparin concentration. In plasma this is 79.5 min^{-1} per I. U. of heparin/ml

Table 4 Specific activities of various heparins

	Total
Standard heparin	13.65
Calciparin	10.33
Enoxaparin	1.70
Fraxiparin	2.36
LMW standard	6.55

The specific activity values are the rate of increase of k_{dec} expressed in $\text{min}^{-1}/\mu\text{M AT III}/(\mu\text{g Hep./ml})$.

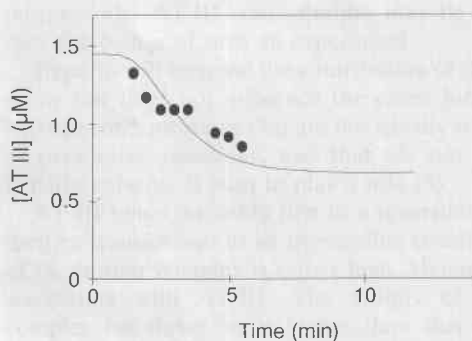


Fig. 4 AT III consumption during the thrombin generation test. ● AT III level calculated from k_1 ; drawn line: calculated level of AT III

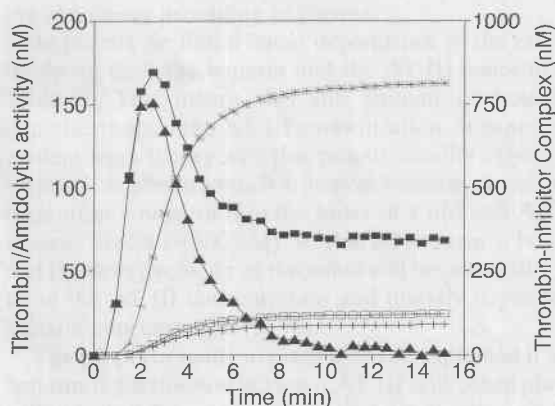


Fig. 5 A thrombin generation curve and the calculated course of different reactants. ■ Experimental data (left scale); ▲ free thrombin (calculated, left scale); + minor inhibitors-thrombin complex (calculated, right scale); □ $\alpha_2\text{M}$ -thrombin complex (calculated, right scale); * AT III-thrombin complex (calculated, right scale)

or 15.44 min^{-1} per $\mu\text{g heparin/ml}$. In serum it is 55.9 min^{-1} per unit heparin/ml or 10.85 min^{-1} per $\mu\text{g heparin/ml}$. Thus in serum it is about 1.5 times lower than that in plasma. This is the same ratio as between the plasma antigenic AT III level and the serum antigenic AT III-level, which, if we admit that only free AT III is revealed by the antibody (see Discussion), suggests that k_1 is proportional to both the AT III concentration and the heparin concentration. Indeed, a second order rate constant (k_1') i.e. k_1 expressed per $\mu\text{M AT III}$, appears to be identical in plasma and serum (Table 2).

V. A Better Approximation of Prothrombinase Activity

In a previous article we have shown that it is possible to obtain the course of the velocity of prothrombin conversion (i.e. the course of the prothrombinase activity) from the course of the

thrombin concentration and the decay constants of thrombin (1). The reasoning behind this calculation is that the net changes of the thrombin concentration are the sum of prothrombin conversion velocity and the thrombin inactivation velocity at any moment. In these calculations we took k_1 as a constant, on the basis of the fact that we could not find a significant change between 4 and 7 min after the start of the reaction. From Fig. 4 it is clear that indeed in this time interval the changes in AT III concentration, and hence of k_1 , are small. Nevertheless, over the whole of a thrombin generation experiment k_1 cannot realistically be represented by a constant. We therefore modified the calculation of the velocity of thrombin decay from $v_{\text{dec}} = (k_1 + k_2) [T]$ to $v_{\text{dec}} = (k_1' [\text{AT III}] + k_2 + k_3) * T$. As in the previous calculation $[T]$ is the concentration of free thrombin at the moment of calculation, obtained from the measured amidolytic activity minus the contribution of the $\alpha_2\text{M}$ -thrombin complex. New is $[\text{AT III}]$, i.e. the concentration of free AT III at the moment of calculation, obtained by subtracting from the initial AT III concentration the amount of AT III bound in the AT III-thrombin complex (see also Appendix). Table 3 shows a comparison of the old and the new way of calculating prothrombinase activities. Although the absolute values differ, the inhibitions brought about by heparin are comparable.

Fig. 5 depicts the course of the various reactants as calculated with the new algorithm. The course of the AT III concentration during coagulation was also calculated from the observed changes in k_1 .

VI. The Second Order Decay Constant of Various Heparins

On the basis of the observation that k_{dec} is proportional to both the concentration of AT III and the heparin concentration, it is a logical next step to express the antithrombin specific activity of heparin in terms of the increase of the thrombin decay constant that it causes at a normalised AT III level.

In Table 4 we show the specific antithrombin activities of several current heparins expressed as the increase of k_1' caused by 1 μg of heparin per ml plasma normalised to an AT III level of 1 μM .

Discussion

At any moment during coagulation, the thrombin decay process can be described as the sum of three inactivation reactions, due to a) AT III, b) $\alpha_2\text{M}$, and c) α_1 -antitrypsin and miscellaneous minor inhibitors. The rate of decay for each of these reactions is given by the product of the thrombin concentration, the inhibitor concentration and the second-order rate constant for the reaction. The concentrations of $\alpha_2\text{M}$ and α_1 -antitrypsin are so high compared to the amounts of thrombin that they bind [$\alpha_2\text{M}$: $3.5 \mu\text{M}$ inhibiting $<0.40 \mu\text{M}$ thrombin; α_1 -antitrypsin: $25 \mu\text{M}$ inhibiting $<0.15 \mu\text{M}$ thrombin (1)], that they may be considered as constant over the course of a thrombin generation experiment. These reactions thus follow pseudo first-order kinetics with a first-order rate constant equal to the product of the inhibitor concentration and the second-order rate constant.

This is not the case for AT III, however. In normal plasma about two thirds of the total amount of prothrombin converted, i.e. $\sim 1 \mu\text{M}$ will end up as thrombin captured by AT III, which accounts for a decrease of AT III from $2.5 \mu\text{M}$ to $1.5 \mu\text{M}$ (Table 2). It is remarkable that the immunodiffusion apparently assesses free AT III only. Probably by the interaction with thrombin and/or by the interaction of the AT III-thrombin complex with vitronectin (13) the antigenic determinants of AT III are masked.

Because of AT III consumption during coagulation, second-order kinetics must be used for the description of the AT III dependent thrombin decay, i. e.:

$$v = k_1' [\text{AT III}] [\text{IIa}]$$

instead of

$$v = k_1 [\text{IIa}].$$

The situation is different when measuring thrombin decay constants. In this type of experiment typically only 100 nM of thrombin will be complexed to AT III so that for all practical purposes the AT III concentration may be considered constant over the course of such an experiment.

Heparin will increase the contribution of the AT III dependent decay but does not influence the other inhibitors (2). Only at heparin concentrations that are not usually reached in therapeutic or preventive situations, and that are not discussed here, will heparin cofactor II start to play a role (3).

AT III binds thrombin first in a reversible complex which can then be transformed to an irreversible covalent complex. The K_d of the former complex is rather high. Heparin acts by reversibly associating with AT III. The affinity of the heparin-AT III complex for thrombin is higher than that of AT III alone by several orders of magnitude. The rate constant for the formation of the irreversible thrombin AT III complex is not altered (14). Thus, the rate of inactivation of thrombin is proportional to the amount of antithrombin-heparin complex under the non saturating conditions prevailing in plasma.

In plasma we find a linear dependence of the rate of thrombin decay on both the heparin and the AT III concentration (Fig. 3, Table 2). This means that the amount of bound heparin is dependent upon the AT III concentration. It is not automatically evident from theory why this proportionality exists. In a purified system it is absent, which is logical because the concentration of high affinity heparin is in the order of 1 nM and AT III is usually in large excess (~500 nM), so that all heparin is bound to AT III and the decay velocity of thrombin will be practically independent upon the AT III concentration and linearly dependent upon the heparin concentration (9, 10).

The proportionality in plasma can be explained if we admit that heparin is partitioned between AT III and other plasma proteins with which it forms complexes that are inactive on thrombin. In that case the amount of AT III present will determine how successfully AT III can compete with other heparin-binding plasma proteins.

Heparin is known to bind to many plasma proteins such as albumin, fibrinogen, fibronectin, lipoproteins. To histidin-rich glycoprotein (HRGP, plasma concentration ~5 µM) it binds with high affinity ($K_d = 7$ nM) (15-17). We surmise that apart from HRGP there may be several more heparin binding proteins responsible for the observed proportionality of heparin dependent thrombin inhibition with the heparin concentration.

The concentration of the AT III heparin complex (C) in the presence of AT III (A) and one other protein (B) binding heparin with a dissociation constant of K_{Db} is given by:

$$C = H_{\text{tot}} \lambda A_{\text{tot}} / (\lambda A_{\text{tot}} + K_{Da}) \quad (\text{I})$$

where

$$\lambda = 1 - B_{\text{tot}} / (B_{\text{tot}} + K_{Db}) \quad (\text{II})$$

in the case of $K_{Da} \gg \lambda A_{\text{tot}}$, as is true for HRGP ($K_{Da} \sim 160$ nM, $\lambda A_{\text{tot}} \sim 2$ nM) equation I reduces to:

$$C = H_{\text{tot}} \lambda A_{\text{tot}} / K_{Da} \quad (\text{III})$$

which states that C , and therefore the AT III dependent thrombin decay velocity, is indeed linearly proportional to the AT III

concentration under the above conditions. Therefore, the interaction of heparin and HRGP alone could explain that the amount of heparin bound to AT III is dependent upon the heparin concentration as well as upon the AT III concentration. This property is not affected by the presence of more proteins binding heparin reversibly with different affinity. Note also that it follows from the above equations that the thrombin decay velocity in the presence of heparin is relatively insensitive to variability in the concentrations of other heparin binding proteins, because already for HRGP alone $B_{\text{tot}} \gg K_{Db}$.

The threshold phenomenon seen in Fig. 3 is most likely explained assuming that a certain low amount of heparin binds (perhaps irreversibly) to some plasma component with a much higher affinity than to AT III. The K_d must be in the sub-nanomolar range, but the concentration, equivalent to 0.01 U/ml heparin, should be low: ~0.35 nM. It remains unexplained why during the clotting process this high affinity plasma component increases. Traces of platelet factor 4 (pf4) might explain the phenomenon in plasma but not the increase observed in serum obtained from platelet poor plasma.

The effect of SBTI on the decay constant of thrombin is most easily explained by a reversible interaction between SBTI and thrombin. This interaction does not involve the active site since SBTI has no influence on the amidolytic activity of thrombin (data not shown). From the fact that the same effect is seen with an AT III and an HC II dependent reaction, and because SBTI is a known inhibitor of serine proteases we presume that it binds to thrombin without impairing its catalytic activity but hindering its interaction with antithrombins. Because the SBTI effect is identical in the presence and in the absence of heparin, it does not affect the conclusions of our previous work.

The proportionality of k_1 with the concentration of AT III has its consequences for the calculation of prothrombinase activity. We previously measured k_{dec} at 2 min after the thrombin peak, when prothrombin conversion had largely stopped but a sufficient amount of thrombin was still present. However, the AT III concentration by that time is already close to its serum concentration and k_{dec} is near its minimum value. k_{dec} is subsequently used to calculate the thrombin decay velocity for the entire thrombin generation curve. This leads to an underestimation of the thrombin breakdown velocity for all but the final part of the thrombin generation curve. Since the prothrombin converting activity is calculated as the sum of the observed rate of change of the thrombin concentration and the calculated thrombin decay velocity, it is underestimated as well. Measuring thrombin decay by addition of exogenous thrombin in non activated plasma which still contains 100% AT III, and counting with the consumption of the AT III concentration during coagulation when calculating thrombin decay rates solves this inaccuracy (cf. Annexe). As expected (Table 3) prothrombinase activities calculated in this way are higher. On the other hand, the relative differences between prothrombinase activities calculated for different experiments remain roughly the same. Thus, the main conclusions from our previous work involving the lack of inhibition of prothrombin conversion in the presence of heparin are not affected. It should be noted that the effect of inhibitor consumption on thrombin decay is even more pronounced in the case of heparin cofactor II/dermatan sulfate, since the concentration of HC II is about 50% of the plasma AT III concentration. Thus HC II/dermatan sulfate dependent thrombin decay may diminish quite dramatically over the course of a thrombin generation experiment. It implicates that the only valid way to measure the effect of dermatan sulfate on thrombin breakdowns is to measure the decay of exogenously added thrombin in non activated plasma.

The modifications to the algorithm for calculating prothrombinase activities from thrombin generation curves are

implemented in the computer programme "ThrombinoScope" (1) which can be obtained from the authors.

The main conclusion from the results presented is that both in the absence and in the presence of heparin the rate of AT III dependent thrombin decay during the course of coagulation in plasma is proportional to the amount of AT III present. This allows to define a standard independent unit of heparin (18): *One standard independent unit (S.I.U.) of heparin antithrombin activity is that concentration of heparin that increases the decay constant of thrombin in plasma by one inverse minute per μM of available AT III.* Or, in other words, one S.I.U. is the concentration of heparin that increases by 1 min^{-1} the AT III dependent second-order decay constant of thrombin in plasma that contains $1 \mu\text{M}$ of AT III. Plasmas as a rule will not contain $1 \mu\text{M}$ of AT III but the results obtained with an arbitrary normal plasma can be expressed in that way by deviding the values found by the AT III content of the plasma. With this unit one can unequivocally express the specific antithrombin activity of a heparin in S.I.U. per unit weight of heparin (Table 4). We are currently performing experiments that show that anti-factor Xa activity can be treated analogously.

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ANNEXE

An Improved Algorithm for the Calculation of Prothrombinase Activity

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Prothrombinase Activity

The rate of change (V) of the thrombin concentration (T) in plasma is defined by the rate of thrombin formation (prothrombinase activity, P) and the rate of thrombin breakdown (D):

$$V(t) = dT/dt = P(t) - D(t). \quad (I)$$

$V(t)$ can be obtained from the measured course of the amidolytic activity. $D(t)$ is determined from the independently measured thrombin decay constants and the thrombin concentration $T(t)$. The prothrombinase activity $P(t)$ is then calculated by summing $D(t)$ and $V(t)$ for each time point.

Thrombin Concentration

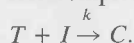
The experimentally determined amidolytic activity E_i at time point t_i reflects the thrombin concentration T_i at this point and the concentration of the α_2 -macroglobulin-thrombin complex MT_i at time t_i :

$$E_i = T_i + fMT_i. \quad (II)$$

Since the α_2 M-thrombin complex has not the same activity towards the chromogenic substrate S2238 as free thrombin, a factor f is necessary. The value of f has been determined to be 0.556.

Thrombin Decay

Thrombin in a plasma is inhibited by a number of serine protease inhibitor proteins I , giving rise to an essentially irreversible complex C :



The rate of formation of C is then given by

$$dC/dt = -dT/dt = k I(t) T(t).$$

Serine protease inhibitors relevant to thrombin breakdown in plasma are Antithrombin III (A), α_2 -Macroglobulin (M), Heparin Cofactor II (H), and a number of serpins with a relatively unimportant anti-thrombin activity (R). The concentration of α_2 M (M) does not decrease significantly during coagulation, nor does the concentration of the group of inhibitors of secondary importance (R). Therefore, it is reasonable to assume first order kinetics

for the inactivation of thrombin by these inhibitors:

$$D(t) = [k_1' A(t) + k_4' H(t) + k_2 + k_3] T(t) \quad (\text{III})$$

with $k_2 = k_2' M$ and $k_3 = k_3' R$.

Procedure

In practice the course of amidolytic activity is measured at discrete time points about 30 s apart. The rate of change of the thrombin concentration at time $i-1$ is estimated as the average rate of change between time $i-1$ and time i :

$$V_{i-1} = (T_i - T_{i-1}) / (t_i - t_{i-1}).$$

Values of the first-order decay constants for thrombin due to $\alpha_2 M$ and due to minor inhibitors, and of the second-order decay constants for thrombin due to AT III and HC II are measured in separate experiments. Prothrombinase calculations are then carried out by a computer program according to the following pseudo code:

an array E contains the measured amidolytic activities
an array t contains the timepoints of measurement of these amidolytic activities

A_0 : user input

H_0 : user input

$MT_0 = 0$

$RT_0 = 0$

$T_0 = 0$

k_1', k_2, k_3, k_4' : user input

for $i = 1$ to number-of-points do
begin

$\Delta t = t_i - t_{i-1}$

$MT_i = MT_{i-1} + k_2 * T_{i-1} * \Delta t$

$RT_i = RT_{i-1} + k_3 * T_{i-1} * \Delta t$

$A_i = A_{i-1} - k_1' * A_{i-1} * T_{i-1} * \Delta t$

$H_i = H_{i-1} - k_4' * H_{i-1} * T_{i-1} * \Delta t$

$T_i = E_i - f * MT_i$

$V_{i-1} = \Delta T / \Delta t = (T_i - T_{i-1}) / \Delta t$

$D_{i-1} = (k_1' * A_{i-1} + k_2 + k_3 + k_4' * H_{i-1}) * T_{i-1}$

$P_{i-1} = V_{i-1} + D_{i-1}$

$O_i = T_i + MT_i + RT_i + A_0 - A_i + H_0 - H_i$

end.

The arrays and variables used represent the following quantities

T : thrombin concentration

E : experimentally determined amidolytic activity

A : AT III concentration

H : HC II concentration

MT : $\alpha_2 M$ -thrombin-complex concentration

RT : other-inhibitors-thrombin-complex concentration

V : delta of thrombin concentration

D : velocity of thrombin decay

P : velocity of thrombin formation (prothrombinase activity)

O : quantity of prothrombin converted

t : time

k_1', k_4' : second-order decay constants for AT III and HC II

k_2, k_3 : first-order decay constants for $\alpha_2 M$ and Rest

Estimation of the α_2 Macroglobulin Dependent Decay Constant

Since the complex of $\alpha_2 M$ and thrombin has residual amidolytic activity, the $\alpha_2 M$ dependent decay constant k_2 can be estimated directly from thrombin generation curves. First, thrombin concentrations are calculated from amidolytic activities using a range of values κ for $f k_2$. These calculated thrombin concentration T_κ are related to the real (but unknown) thrombin concentrations T in the following manner:

$$T_\kappa(t) = T(t) + (f k_2 - \kappa) \int_0^t e^{-\kappa(t-s)} T(s) ds. \quad (\text{I})$$

If it is assumed that prothrombin conversion has stopped at a timepoint t_0 then

$$T(t) = T(t_0) e^{-(k_1 + k_2)(t-t_0)}, \quad t \geq t_0. \quad (\text{II})$$

If it is further assumed that $T(t) = 0$ after t_0 , and that $k_1 + k_2 \gg \kappa$, then it follows from (I) and (II) that:

$$T_\kappa(t) \approx (f k_2 - \kappa) C e^{-\kappa(t-t_0)} \quad (\text{III})$$

where C is the following constant:

$$C = \int_0^{t_0} e^{-\kappa(t_0-s)} T(s) ds.$$

Thus, $T_\kappa(t) / e^{-\kappa(t-t_0)}$ is a constant value for a given κ after timepoint t_0 . $T_\kappa(t) / e^{-\kappa(t-t_0)}$ is then plotted as a function of κ , and k_2 is determined from the intersection of this function with the horizontal axis.